UNDERSTANDING THE SYNERGISTIC ACTION OF SERUM INFLAMMATORY MARKERS AND GROWTH FACTORS PROFILE IN EXCISIONAL WOUND HEALING PHASES IN RATS' EXPERIMENTAL MODEL INTRA-PERITONEALLY TREATED WITH ECHINODERMATA GLYCOSAMINOGLYCANS (GAGS)

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by

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF DIAGRAMS

LIST OF ABBREVIATION

S. vastus	*Stichopus Vastus*
GAGs	Glycosaminoglycans
SD	Sprague Dawley
PBS	Phosphate Buffer Saline
mg	Milligram
kg	Kilogram
ELISA	Enzyme-Linked Immunosorbent
qRT-PCR	Real Time Polymerase Chains Reaction
ECM	Extra Cellular Matrix
S. hermanni	*Stichopus hermanni*
ACE	Angiotensin I Converting Enzyme
IL-6	Interleukin-6
IL-10	Interleukine-10
MMP-9	Matrix metalloproteinases-9
VEGF-A	Vascular Endothelial Growth Factor
TNF-α	Tumor Necrosis Factor Alpha
PDGF-A	Platelet-Derived Growth Factor-A
TGF-β1	Transforming Growth Factor Beta 1
BSE	Bovine Spongiform Encephalopathy
TSE	Transmissible Spongiform Encephalopathy
FMD	Foot and Mouth Diseases
CS	Chondroitin Sulphate
DS	Dermatan Sulphate
KS	Keratan Sulphate
HS	Heparan Sulphate
HP	Heparin
HA	Hyaluronan
M1	Macrophages
FN	Fibronectin

SPARC	Secreted Protein, Acidic and Rich in Cysteine
EGF	Epidermal Growth Factor
IGF-1	Insulin-Like Growth Factor-1
NETs	Neutrophil Extracellular Traps
ROS	Reactive Oxygen Species
M2	Macrophages
FGF	Fibroblast Growth Factor
PDGF-B	Platelet-Derived Growth Factor-B
CD4+	T-Helper Cell
CD8+	T-Suppressor-Cytotoxic Cells
EDA	Extra Domain-A
IL-6Ra	Interleukin-6 Receptor
STAT-JAK	Signal Transduction and Activator of Transcription- Janus Kinase
IL-1	Interleukin-1
sTNF	Monomer TNF
tmTNF	Monomeric Type 2 Transmembrane Precursor Protein
PIGF	Placental Growth Factor
Ag-Ab-E	Antigen-Antibodies-Enzyme
BSA	bovine serum albumin
DNA	deoxyribonucleic acid
RNA	ribonucleic acid
mRNA	messenger RNA
ssDNA	single strand deoxyribonucleic acid
dNTP	Deoxynucleotides
$MgCl_2$	magnesium chloride
dsDNA	Double strand deoxyribonucleic acid
RT-PCR	Reverse Transcription PCR
PCR-Q-PCR	Quantitative PCR
SNPs	single-nucleotide polymorphisms
RAPD	Random Amplification of Polymorphic DNA PCR
GMO	genetically modified organisms

CT	threshold cycle
ACT	ACTIN
GAPDH	Glyceraldehyde-3-phosphate
EF1a	elongation factor 1-alpha
TUBa	alpha tubulin
TUBb	tubulin beta-1 chain
UBQ	ubiquitin
UBC	ubiquitin-conjugating enzyme
CAC	clathirin adaptor complexes medium subunit
CYP	cyclophilin
GLU	endo-1,3-betaglucanase
MDH	malate dehydrogenase
TIP41	tonoplast intrinsic protein
NTB	nucleotide tract-binding protein
NaCl	Sodium chloride
PBS	phosphate buffer saline
H&E	Haematoxylin and Eosin
PRFM	platelet-rich fibrin matrix

MEMAHAMI TINDAK BALAS SINERGIS PENANDA KERADANGAN SERUM DAN PROFIL FAKTOR PERTUMBUHAN KETIKA FASA PENYEMBUHAN LUKA EKSISI PADA MODEL TIKUS PENYELIDIKAN YANG DIRAWAT SECARA INTRA-PERITONEAL DENGAN GLYCOSAMINOGLYCANS (GAGS) ECHINODERMATA.

ABSTRAK

Stichopus vastus; adalah invertebrata timun laut yang kaya dengan glycosaminoglycans (GAGs). Dikategorikan sebagai species Gamat, telah lama diguna pakai dalam terapi tradisional untuk rawatan luka. Kajian ini direka untuk mengkaji kesan GAGs yang diekstrak daripada dinding integumen *S. vastus* untuk rawatan luka pada tikus secara suntikan intra-peritonium. Dua puluh empat (24) ekor tikus jantan Sprague Dawley, dibahagikan kepada empat kumpulan (n=6) iaitu kumpulan pemalar yang diberi garam penampan fosfat (PBS) dan tiga kumpulan dengan rawatan GAGs yang terbahagi mengikut berat badan: 2mg/kg, 4mg/kg dan 6mg/kg, Setiap tikus penyelidikan dengan berat badan: 250–400gram disuntik dengan GAG setiap hari selama 12 hari. Sampel lapisan kulit dan darah diambil untuk penilaian makroskopik dan mikroskopik, ekspresi protein dan gen dari penanda bio terpilih (IL6, IL10, MMP-9, TNF-α, TGF-β1, VEGF-A, PDGF-A) telah dianalisa menggunakan ELISA dan qRT-PCR. Dalam kajian makroskopik, tiada perbezaan yang signifikan ($p>0.008$) wujud dalam semua kumpulan. Sementara, untuk kajian mikroskopik, tiada perbezaan yang signifikan ($p>0.008$) wujud antara kumpulan berhubung kait dengan proses epitelialisasi, keradangan, pertumbuhan fibroblast, organisasi serat gentian kolagen dan pembentukan salur pembuluh darah baru. Walaubagaimanapun, kajian ini menunjukkan, bahawasanya pada dos 2mg/kg atau

4mg/kg GAGs daripada *S. vastus*, kedua dos secara peritoneum dapat mempercepatkan proses penyembuhan luka di fasa awal penyembuhan luka apabila disiasat secara pemeriksaan makro dan mikroskopik. Untuk studi ekspresi protein, kajian menunjukkan wujudnya perbezaan signifikan ($p<0.05$) antara kumpulan rawatan, terutamanya dalam penanda bio: IL-6, IL-10, MMP-9, VEGF-A dan TNF-α, manakala, tiada perbezaan yang signifikan wujud untuk penanda bio: PDGF-A dan TGF-β1. Sementara itu, studi ekspresi gen menunjukkan bahawa tiada perbezaan signifikan ($p>0.05$) dalam semua penanda bio terpilih. Berdasarkan data dari kajian ini, ekspresi protein dan gen menunjukkan, bahawasanya 2mg/kg GAGs mempengaruhi penanda bio: IL-6, IL-10, MMP-9 dan VEGF-A, manakala 4mg/kg GAGs mempengaruhi penanda bio: TNF-α, PDGF-A dan TGF-β1. Kepekatan ekstrak GAGs secara selektif mempengaruhi dan memberi kesan kepada penanda bio, tatkala proses penyembuhan luka itu sendiri adalah suatu proses yang kompleks. Ekstrak GAGs daripada *S. vastus* berpotensi untuk bertindak sebagai dos penggalak pro-keradangan, anti-keradangan, anti-mikrobial dalam penyembuhan luka dengan mensasarkan sitokin, kemokin dan enzim yang terlibat dalam proses penyembuhan luka. Dalam kajian ini, kepekatan ekstrak GAGs diperhatikan mempunyai kesan selektif kepada aktiviti biomarker, bagaimanapun, kerana fasa penyembuhan luka itu sendiri sebenarnya merupakan proses yang kompleks, laluan mekanisme ini yang terdiri daripada interaksi sitokin, chemokines dan enzim mesti disiasat dengan lebih teliti untuk kajian terperinci.

Kata Kunci: Penyembuhan luka, Glycosaminoglycans, *Stichopus*

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ABSTRACT

Stichopus vastus; sea cucumbers, is a marine invertebrate which is rich in glycosaminoglycans (GAGs). This sea cucumber has long been utilised as traditional therapy alternatives, especially as wound healing remedy. The current study is designed in order to explore the effect of GAGs from integument wall of *S. vastus* species for wound healing properties in rats by intra-peritoneal injection with GAGs extract daily. Twenty-four (24) male Sprague Dawley rats were divided into four groups (n=6) which are control group; given phosphate buffer saline (PBS) and three treated groups of GAGs; at 2mg/kg, 4mg/kg and 6mg/kg body weight, respectively. Rats weight from 250-400 grams were treated with GAGs daily for 12 days. Skin graft and blood samples were taken for macroscopic and microscopic evaluation; while protein and gene expression of selected biomarkers (IL6, IL10, MMP-9, TNF-α, TGF-β1, VEGF-A, PDGF-A) were analysed with ELISA and qRT-PCR, respectively. In the macroscopic study, there was no significant difference ($p>0.008$) noted between all groups. Meanwhile, in the microscopic study, there were also no noted significant difference ($p>0.008$) between the groups in epithelialization, inflammation, fibroblast proliferation, collagen fibers organization and new vessels formation. However, via this research activities, it was revealed that, both either 2mg/kg or 4mg/kg of extracted glycosaminoglycans (GAGs) from *S. vastus* can enhance wound healing process at early phase of the wound healing when investigated macro and microscopically. In addition, in protein expression, the study disclosed that, there were significant difference ($p<0.05$) between treatment and control groups in IL-6, IL-10, MMP-9, VEGF-A and TNF-α while, there were no significant difference in PDGF-A and TGF-β1. On the other hand, gene expression study showed that, there were no significant difference ($p>0.05$) in all selected biomarkers. All in all, protein expression and gene expression revealed that, 2mg/kg of GAGs effects biomarkers: IL-6, IL-10, MMP-9 and VEGF-A, while 4mg/kg of GAGs effects biomarkers: TNF-α, PDGF-A and TGF-β1. GAGs extraction concentration selectively effects these biomarkers, as the wound healing cascade itself in a complex process. Extracted glycosaminoglycans (GAGs) from *S. vastus* have the potential to act as promoter for pro-inflammatory, anti-inflammatory, anti-microbial effect on wound healing by targeting cytokines, chemokines and enzyme that participate in wound healing cascade to execute their pathophysiological roles. In this study the GAGs extraction concentration was observed to have selectively effects the biomarkers activities, however, as the wound healing cascade itself is actually a complex process, the pathway of this mechanism which consist of interactions of cytokines, chemokines and enzyme must be carefully investigated for further detail study.

Keywords: Wound healing, Glycosaminoglycans, *Stichopus*

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Sea cucumbers are marine creatures, grouped as invertebrate from the class Holothuroidea (Masre *et al.*, 2012; Pangestuti & Arifin, 2018). Anatomically the body of these sea cucumber contains a single branch gonad. As such the body is elongated in structure and well covered with an indurated epithelial skin feature. This marine invertebrate, wild-life population biodiversity within the Asia Pacific region is purported to be of about 1716 species (Pangestuti & Arifin, 2018). In Malaysia, sea cucumber is famous, and well exploited. However, a group of the sea cucumbers species have been categorized as ‘Gamat’, especially species within the family of Stichopodidae. Various local signature names for these sea cucumbers existed. Such ‘timun laut’, ‘bat’, ‘balat’ and ‘brunok’ whilst the Chinese community refers sea cucumber as sea ginseng or ‘hoi sum’ or ‘hai shen’ because of their healing properties (Kamarudin *et al.*, 2015). *Stichopus vastus spp*. is known by other name such as brown curry or ngimes (Masre *et al.*, 2012), Gamat batu or Gamat kiulu (Kamarudin *et al.*, 2015).

Sea cucumbers are famous to be consumed as food sources especially in Asian. Besides, they are also well known as traditional remedies as they are said to be effective against many diseases especially hypertension, asthma and rheumatism. In addition, they are good for cuts and burns, impotence and constipation (Bordbar *et al.*, 2011). *Stichopus vastus* has also been reported to act as anti-cancer, radical scavenging and it has wound healing properties (Rasyid, 2018). Moreover, it is also be used as functional ingredient in nutraceuticals, cosmetics and food products as it is rich with collagen in its integument tissue (Wibowo *et al.*, 2019).

Wound healing occurs after physiological damage of skin and highly organized process in repairing wound by replacing and regenerating tissue and cells to restore the functional tissue integrity (Melrose, 2016; Tottoli *et al.*, 2020). Wound is divided into two classes which are chronic and acute. The acute wound usually involving epidermis and superficial dermis such as surgical incision, thermal wounds, abrasion and laceration associated with being infected (Dreifke *et al.*, 2015). The wound healing process involved several complexes and overlapping phases which includes hemostasis, inflammatory phase, proliferation phase and remodelling phase (Pauzi *et al.*, 2018). Hemostasis phase take over straight away after the injury occurs which initiate the intrinsic and extrinsic clotting cascade (Sinno & Prakash, 2013). The damage tissue leads to vasoconstriction and platelet aggregation to control bleeding at the site of injury by forming fibrin clot and in an instance prevent more blood lost due to injury (Ghatak *et al.*, 2015; Sinno & Prakash, 2013). In addition, the injured tissue release growth factor, cytokines and chemoattractant which eventually attract inflammatory cells such as neutrophil, lymphocytes and macrophages (Avishai *et al.*, 2017).

The infiltration of neutrophil; earliest inflammatory cells, to the site of injury begins during the inflammatory phase. Neutrophil acts as the first defence cell able to kill the bacteria, exposed due the tissue damage and at the same time promote the wound healing process (Ghatak *et al.*, 2015; Pereira & Bártolo, 2016). Meanwhile when the proliferation phase takes over, the macrophages initiate phagocytosis of all foreign organism as well as the accumulated neutrophils while stimulating keratinocytes, fibroblasts and the angiogenesis proses for tissue regeneration (Ghatak *et al.*, 2015; Sorg *et al.*, 2017). The dynamics of wound healing event reaches its final stages as the remodelling phase. During this stage, the tissue structure is remodelled and renovated to adapt to their new condition (Nour *et al.*, 2019). Wound would heal to some extent as it left degrees of collagenous scar formation which undergoes maturation in the process and at the same time will never return to its original structure and somehow hinder the original function of the tissue (Melrose, 2016). In other cases, when wound healing process do not proceed smoothly, it can come to a standstill which lead to potential dysfunction; chronic wound (Dreifke *et al.*, 2015). This condition can lead to organ failure and in worst case can lead to death, if not well treated (Pereira Beserra *et al.*, 2019).

According to (Dreifke *et al.*, 2015), the increasing of occurrence of diabetes and obesity can cause a simple wound to be a chronic wound and these leads to increase needs for wound care management. The cost for wound care for individual with diabetes and obesity has risen dramatically and leads to financial burden. In order to counter and solve the problem, many alternatives have been found and some still in research phases. From aforementioned advantages of sea cucumber, the healing properties is at the centre of attention as it could be beneficial in wound care area. The compound which help in wound healing process is called glycosaminoglycans (GAGs) also known as mucopolysaccharides, plays a huge role in wound healing process by giving countless physiological events which take part in myriad biological process; cell adhesion, cell migration, tissue repair, ECM assembly, cell signalling and immune response (Kamhi *et al.*, 2013; Pauzi *et al.*, 2018; Yamada *et al.*, 2011a).

1.2 Justification of study

This study is conducted as a transpired continuation of previous study by (Masre *et al.*, 2012). Previously, the comparation of extracted GAGs compounds was from 2 species of locally harvested sea cucumbers which were the: *S. hermanni* and *S. vastus*. In that study, *S. vastus* integumental wall was quantitated to contained more sulphated GAGs, able to enhanced the wound healing process in animal model. The quantified extract is more dependable as compared to those extracted from *S. hermanni*. Thus, further research needs to be done to find out more about the roles of GAGs in assisting wound healing process. The study was done on rat as wound model and was evaluated via macroscopic and microscopic investigations as well as to find out the involvement of inflammatory markers which were cytokines, growth factors, chemokines in wound healing cascade via biochemical test (ELISA) and gene expression. The research concern on freshly harvested Malaysian sea cucumber, *S. vastus* (Sluiter 1887), from which contained GAGs extracted from integument body wall. This Stichopodidae family has been chosen as they dominate the Malaysia coastal area, hot shallow-water tropics to the warm temperate region. *S. vastus* can be found in an indigenous commensal invertebrate of the coastal areas in Terengganu, Malaysia.

This research undertaking may potentially provide a relevant impact to clinical understanding revolving the concern on wound healing cascade as well as potentially elucidate, explore traditional remedies preparations of local sea cucumber which contains GAGs especially those in *S. vastus* to orchestrate and enhanced role in wound healing processes.

1.3 Objectives of Study

1.3.1 General Objective

To understand the mechanism of marine sourced GAGs in aiding and harnessing wound healing pathophysiology process in experimental animal models such as its novel to extrapolate and understand how by identifying the fundamental involvement and relationship of serum inflammatory markers, and growth factors associated with the said wound.

1.3.2 Specific Objectives

a. To compare the rate of wound healing dynamics of different concentration of GAGs
b. To investigate the microscopical effect of GAGs in wound healing
c. To study the expression level of cytokines and growth factors in wound healing following intraperitoneal injection of GAGs in experimental rat model
d. To analyze the expression of selected genes related mediators to wound healing after treatment with *Stichopus vastus* (GAGs)

1.4 Hypothesis

Microscopic characteristics of wound healing affected by marine-sulphated GAGs extract are functionally connected to the expression of serum pro-inflammatory and anti-inflammatory cytokines and growth factor genes. Such a wound mechanism causes the expression of the chosen gene combination to accumulate collectively and overpower the healing response.

CHAPTER 2

LITERATURE REVIEW

2.1 Sea Cucumber

Sea cucumbers are marine creature which are grouped as invertebrate from the class Holothuroidea (Masre *et al.*, 2012a; Pangestuti & Arifin, 2018). These marine invertebrates are related to starfish and sea urchins where they are characterized with soft-bodied and tube-shaped like a worm (Masre *et al.*, 2012a; Pangestuti & Arifin, 2018). The body of sea cucumber contains a single branch gonad which make the body elongated and covered with leathery skin. These organisms are greatest in biodiversity population are recorded to be at its greatest taxonomy counts within the Asia Pacific region with 1716 interspecies count (Pangestuti & Arifin, 2018). Altogether these sea cucumbers are common habitats are: within shallow coasts and around coral reefs to in the deep-sea bottoms. Their mobility is slow and are said to have a long-life span at the average age of 5 to 10 years (Yuniati & Sulardiono, 2019). Sea cucumbers feed on waste deposited at the bottom of the sea, microscopic algae and dead organic matter and they act as natural recycling machine (Aydin, 2019; Siahaan *et al.*, 2017). They maintain and at the same time improve sediment health by re-stirring up sediment layers that already done by other organisms. Moreover, it can increase the alkalinity and help to protect and prevent coral reef from acidification of surrounding seawater (Che Ghazali, 2019). As aforementioned, sea cucumbers are organisms that deposit-feeders as in they take organic detritus mixed with sand and they defaecate sand which is less organic than what they consumed. The natural recycling machine such as sea cucumber digest organic nitrogen-rich compounds from sea sand and convert to inorganic forms which later can be taken by primary producers (Purcell *et al.*, 2016).

Sea cucumbers act as prey in food chain and are consumed by diverse predators including human and other marine organisms; 19 species of sea star, 17 species of crustaceans, several gastropods, and around 30 species of fishes (Purcell *et al.*, 2016). They actually possess defence mechanisms called as evisceration in which they expel out their internal organ such as intestine and respiratory tree if they are stress or under pressure. The missing organs can be regenerated within 7 days (Li *et al.*, 2018). Besides, they also possess chemical defence in which they expel saponins and terpenes and sticky Cuvierian tubules to deter predators (Purcell *et al.*, 2016). As an option they usually offer their internal organ in place of others body part to their predators to avoid being killed. (Li *et al.*, 2018). The sea cucumbers are consumed and the energy from microalgae and organic detritus that are taken and digested by the sea cucumber produce higher nutrition levels so that, the nutrition is transferred indirectly to the consumers and others organisms (Purcell *et al.*, 2016).

In Malaysia, sea cucumber is famous with the name called 'Gamat' and it is used for all species of sea cucumber within the family of Stichopodidae. Others local peoples also call sea cucumber with 'timun laut', 'bat', 'balat' and 'brunok' whilst Malaysian Chinese community refers sea cucumber as sea ginseng or in Chinese language also known as 'hoi sum' or 'hai shen' because of their healing properties (Kamarudin *et al.*, 2015). In Indonesia, Sea cucumbers are known as 'teripang' or 'trepang', French calls them as 'beche-de-mer' which is translated as food product and in Chamorro, it is known as ‘balate’ (Pangestuti & Arifin, 2018). Sea cucumbers are famous to be consumed as food sources especially in Asian countries. Besides, they are also well known as traditional remedies as they are said to be effectives against many diseases especially hypertension, asthma and rheumatism. In addition, they are good for cuts and burns, impotence and constipation (Bordbar *et al.*, 2011).

2.1.1 Taxonomy of Sea Cucumber

Kingdom: Animalia

Phylum: Echinodermata

Class: Holothuroidea

Subclasses: Dendrochirotacea, Aspidochirotacea, and Apodacea

Orders: Apodida, Elasipodida, Aspidochirotida, Dendrochirotida, Molpadida and Dactylochirotida

Family: Stichopodidae and Holothuriidae

Genus: Stichopus spp.

Taxonomy of sea cucumber (Bordbar *et al.*, 2011; Li *et al.*, 2018; Mondol *et al.*, 2017)

Sea cucumbers belong to Kingdom of animalia where they have the ability to produce saponin though the production of saponin among animalia is rare (Li *et al.*, 2018). They have spiny-skinned so that, they are grouped in phylum of Echinodermata under Holothuroidea class. Then, they are divided to subclasses which are known as

Dendrochirotacea, Aspidochirotacea, and Apodacea. The subclasses are divided by distinguishing their oral tentacles (Bordbar *et al.*, 2011). They are further divided to 6 orders which are Apodida, Elasipodida, Aspidochirotida, Molpadida, Dendrochirotida and Dactylochirotida (Mondol *et al.*, 2017).

Recently, the new revision regarding the taxon of sea cucumbers has been done by (Miller *et al.,* 2017). They further classify Holothuroidea into two main clades or subclasses known as Apodida and Actinopoda. Later, within Actinopoda, it is fractioned into Pneumonophora and Elasipodida. Pneumonophora is separated to another two smaller clades recognise as Holothuriida and Neoholothuriida; Molpadida, Persiculida, Synallactida and Dendrochirotida. In Malaysia, there are two most-famous families of sea cucumber which are known as Stichopodidae and Holothuriidae (Masre *et al.*, 2012a). Stichopodidae is the daughter of order of Synallactida while Holothuriidae is from order of Holothuriida (Miller *et al.*, 2017).

2.1.2 Sea Cucumber Stichopus vastus

Figure 2.1 Sea cucumber Stichopus vastus

Kingdom: Animalia

Phylum: Echinodermata

Subphylum: Echinozoa

Class: Holothuroidea

Subclass: Actinopoda

Order: Synallactida

Family: Stichopodidae

Genus: Stichopus

Species: *Stichopus vastus*

Taxonomy of sea cucumber *Stichopus vastus*, adapted from World Register of Marine Species, 2010.

Sea cucumbers; *Stichopus vastus spp.* is common to be found in seashore region in Malaysia (Abedin *et al.*, 2014). It is also known by other name such as brown curry or ngimes (Masre *et al.*, 2012a), Gamat batu or Gamat kiulu (Kamarudin *et al.*, 2015). Stichopus spp are very similar between species and sometime very deceptive even individually within the same species, hence the identification by their external appearance somehow difficult (Woo *et al*, 2015). However, *S. vastus* can be differentiated with other Stichopus species by their characterization of green and yellow harlequin pattern and rigid body with quadrangular section (Masre *et al.*, 2012a).

This species is not effectively used other than their stomach and intestine, as it is used as raw salad. The remaining part of sea cucumbers including their collagen-rich integument wall mostly are thrown away in some Asian countries (Abedin *et al.*, 2014). In Indonesia, *S. vastus* can also be found in the islands of Karimunjawa and Palau but they are non-commercial biomass as it was not exploited economically and optimally (Yuniati & Sulardiono, 2019).

2.1.3 Nutritional and Medicinal Values of Sea Cucumber

Sea cucumbers have been used as food source in many countries for centuries and most famous in some parts of Asia as a culinary delicacy. They are consumed as they have many biomedicine properties to the consumer. In addition, they give high value-added nutritional compounds in which will be beneficial to health and can be used as functional ingredients that can be added for food and biomedicine production process. The sea cucumbers are thought to boost immune systems and having aphrodisiac effect to the consumers. Sea cucumbers consist of many bioactive compounds especially triterpene glycosides (saponins), chondroitin sulphates, glycosaminoglycan (mucopolysaccharides), sulphated polysaccharides, sterols (glycosides and sulphates), phenolics, peptides, cerebrosides and protein (lectins, collagens, gelatine), vitamin A (carotenoids), fatty acids, riboflavin, niacin, calcium, iron, magnesium, zinc, low sugar and fat content, and no cholesterol (Aydin, 2019; Pangestuti & Arifin, 2018; Siahaan *et al.*, 2017). They are also said to be effectives in biological and pharmacological activities as they act as anti-angiogenic, anticancer, anticoagulant, anti-hypertension, antitumor, anti-inflammatory, antimicrobial, antioxidant, antithrombotic, and wound healing. These pharmacological properties are found in sea cucumbers corresponding to aforementioned bioactive compounds found in different species of sea cucumbers (Bordbar *et al.*, 2011).

Narrowing down to a species of sea cucumbers, *Stichopus vastus* biomass has been reported to be able to act as anti-cancer agents, with radical scavenging abilities and functional in wound healing properties (Rasyid, 2018). Moreover, it is also can be used as functional ingredient in nutraceuticals, cosmetics and food products as it is rich with collagen in the integument tissue (Wibowo *et al.*, 2019). Bioactive compound from collagen in *S. vastus* can be exploited for the production of protein hydrolysates which is consist of small peptides, thus it is enrich with antioxidant and antihypertensive activities (Abedin *et al.*, 2014). Antioxidant is a compound to be at the centre of attention as it plays crucial roles in helping to reciprocate the oxidative stress in human tissues (Silva *et al.*, 2011). Besides, it can give an activity of radical scavenging as it contains bioactive peptides which it inhibits angiotensin, I converting enzyme (ACE) (Wibowo *et al.*, 2019), In an instance can fight against various diseases such as chronic inflammation, atherosclerosis, cancer, cardiovascular disorders, and ageing processes (Silva *et al.*, 2011). For acknowledgement, collagen are found abundant in nature, this statement is especially true, to collagen collections within bovine and porcelain, that have long been used in wound healing managements. However, the outbreak of bovine spongiform encephalopathy (BSE), transmissible spongiform encephalopathy (TSE) and foot and mouth disease (FMD) happen to limit their uses. Therefore, another alternative which is by using collagen from marine organism has secured an attention by researchers (Felician *et al.*, 2019). Pauzi *et al.*, (2018), has mentioned, besides famous of being rich in collagen, *S. vastus* also rich in glycosaminoglycans (GAGs) which also recognise as mucopolysaccharides.

2.2 Glycosaminoglycans (GAGs)

Glycosaminoglycans (GAGs) can be found within the animal Kingdom, not only found in vertebrates but also found in many invertebrate's animals. The GAGs are a component of the both the intracellular matrices and extracellular matrices of animals' anatomical tissues (Kamhi *et al.*, 2013; Yamada *et al.*, 2011b). GAGs take place as proteoglycans (PGs) where the long-chain, unbranched polysaccharides chains are covalently linked to a core protein and can be found on the cell surface of both intra and extracellular matrix (Dantas-Santos *et al.*, 2012; Kamhi *et al.*, 2013; Köwitsch *et al.*, 2018). GAGs consist of repeating disaccharides that is linked by glycosidic bonds form a complex structure (Köwitsch *et al.*, 2018). It unveils unusual structural variability according to tissue and species (Dantas-Santos *et al.*, 2012), thus gives countless physiological events which plays as a central role in myriad biological process; cell adhesion, cell migration, tissue repair, ECM assembly, cell signaling and immune response (Kamhi *et al.*, 2013; Yamada *et al.*, 2011b).

Chondroitin sulphate (CS), dermatan sulphate (DS), keratan sulphate (KS) heparan sulphate (HS) and heparin (HP) are derivatives of GAGs which have been group into sulphated GAGs while only hyaluronan (HA) is of the non-sulphated GAGs group (Table 2.1). Sulphated GAGs have been investigated and reported to be with numerous biochemical functions abilities which give of benefits to human wellness (Masre *et al.*, 2012a)

Table 2.1 Biochemical structure of GAGs. (The table was adapted from Mende *et al.*, 2016)

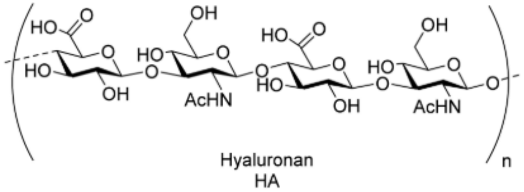
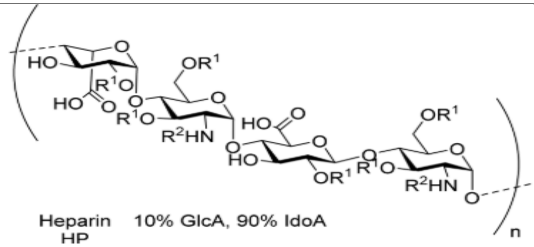
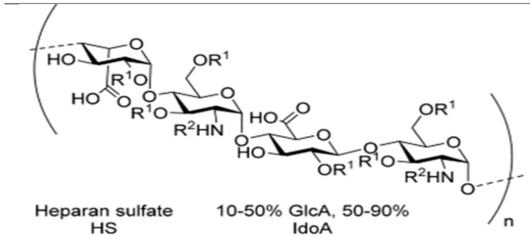
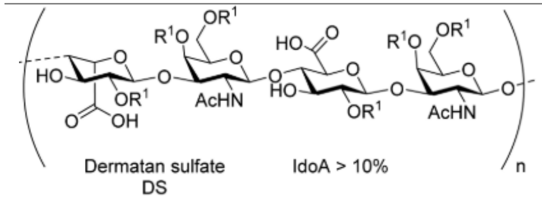
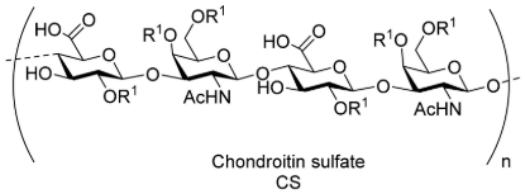
Disaccharides unit of GAGs	Explanation
	**Hyaluronan (HA)** consists of repeating disaccharide which includes D-Glucoronic acid [GlcAβ (1→3)] glycosidically linked to D-N-Acetylglucosamine (GlcNAc). Each of the subunits are linked via β (1→4) glycosidic linkages.
	**Heparin (HP)** consists of repeating disaccharides includes of α,β(1→4) uronic acid; consists of 10% D-Glucuronic acid (β-D-GlcA) and 90% of its C5-epimer L-Iduronic acid (α-L-IdoA), being glycosidically linked to D-N-Acetylglucosamine (GlcNAc) residues and are connected via α (1→4) glycosidic linkages. HP is actually the HS with the highest N- and O-sulfation degree.
	**Heparan Sulfate (HS)** consists predominantly with 10−50% of D-Glucuronic acid (β-D-GlcA) and the appropriate rest of L-Iduronic acid (α-L-IdoA) and these structures can be very in some extent.

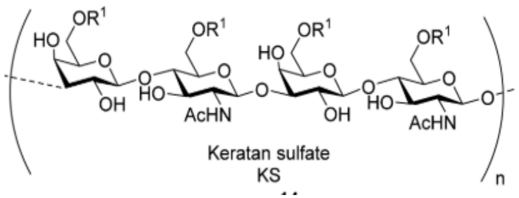
Table 2.1 continued



Dermatan Sulfate (DS). β(1→3)- D-N-Acetylglucosamine (GalNAc) acts as basic neutral sugar component and link to copolymer of iduronic acid (L-IdoA)



Chondroitin Sulfate (CS) consists of copolymers made up of dimeric subunits that are composed of D-glucuronic acid (D-GlcA) and are β(1→3)- linked to N-acetyl-D-galactosamine (D-GalNAc) that is β(1→ 4)-linked to the first monosaccharide in turn so the disaccharide consists of a repeating unit [4)-β-GlcA-(1→3)-β- GalNAc-1→].



Keratan Sulfate (KS) involved of the repeating linkage of β (1→3)- linked sulfated 3 galactose to D-N-Acetylglucosamine (Gal-β-(1,4)-GlcNAcβ1); N-acetyllactosamine subunit.

2.2.1 GAGs from Marine Invertebrates

Glycosaminoglycans (GAGs) can be found in numerous invertebrates and vertebrate animals inland or in ocean. In marine invertebrates, they are said to rich in sulfated polysaccharides with different structures known as sulfated fucan and galactans (Silva *et al.*, 2011). GAGs are different in marine animals to those of terrestrial organisms, mainly in terms of molecular weight and sulfation (Valcarcel *et al.*, 2017). There are few reports that bacteria from inland synthesize Sulphated GAGs while there are no findings in plants, fungi or virus (Silva *et al.*, 2011).

Table 2.2 The comparison between inland and marine extracted GAGs (The table was adapted from (Hanim Zainudin *et al.*, 2014).

Inland Vertebrates	**Marine Invertebrates**
Heterogenous structure	Homogenous structure
Diverse sulfation pattern	Sulfated total, N- and O- sulfated Glycosaminoglycans
Mutational defects in most genes-biosynthetically derived enzyme which cause severe consequences	Stable expansion of sulfated structure: Pharmacologically active compounds are associated to a hetero undescribed compound (reaction to waste to benefits opportunities
Risk for the present of infection	No alteration in structure (morphologically undefended)
Can reliable of availability (cost, volume) restricted to certain use.	Originally species specific

2.2.2 Benefits of GAGs on Wound Healing

Glycosaminoglycans (GAGs) are said to be benefits for wound healing process as they have wound healing properties (Rasyid, 2018). Aforementioned, GAGs consist of sulphated GAGs and non-sulphated GAGs. Sulphated GAGs are chondroitin sulphate (CS), dermatan sulphate (DS), keratan sulphate (KS) heparan sulphate (HS) and heparin (HP) while only hyaluronan (HA) is non-sulphated GAGs.

HA plays dual roles in blood vessel formation by either suppress or enhance angiogenesis depending on the types of HA activated. In addition, HP and HS are responsible to suppress proteolytic degradation upon binding with growth factor and regulate cell adhesion, tumor development and metastasis, brain development, inflammation and enzymes. Beside of their function as anticoagulant, they are also used to treat cancer, pregnancy complications or renal function loss. CS is benefit to numerous biological functions in which it is involve in cell-cell recognition process, anti-inflammatory effect and brain development. Moreover, it can act as therapeutic agent against osteoarthritis and it is able to form interaction between growth factor, cytokines and adhesion molecules. DS has potential as anti-oncogenic and antithrombotic, and also responsible as growth factor regulations. Lastly, KS is assigned solely for tissue hydration (Mende *et al.*, 2016).

In wound healing studies, according to Melrose (2016), they have identified four GAGs which are involved in woung healing cascade. These four GAGs include HA, CS, KS and HS are composed with repeating disaccharides with different chemical structure at carbon -2, -3, -3 or -6 and widely spread in connective tissue.

However, as mentioned earlier by (Mende *et al.*, 2016), we can say that, every division of GAGs can constribute to wound healing process.

2.3 Wound Healing

According to the statistic provided by Wound care Market (2021), it is estimated around 2.5 million peoples in the United State develop a pressure ulcer and about 17.2 million hospitals visit are due to wounds including outpatient and inpatient surgical visits around the globe. Wounds can be classified into acute and chronic as they are depending on how wounds are created such as namely surgery, injuries, extrinsic factors; pressure, burns and cuts, or other pathological conditions; diabetes or vascular diseases (Tottoli *et al.*, 2020).

Wounds are actually a state of physical damage towards tissues or organ systems. Such damages could detrimentally involve separation of epidermal skin layers, thus needing a clinical approach management to revive anatomical properties (Shanthi Kumari *et al.*, 2020). Wound repair or wound healing is a process to replace and regenerate damaged cells, tissues or organ to bring back their normal functions (Melrose, 2016). The abilities to repair or healing are depending on how serious the damage to the tissue happened to be and usually for acute wounds the processes are well organized and going through appropriate process resulting in restoration of anatomical and functional integrity whilst, in opposition, the chronic wounds are unable to reach their optimal anatomical and functional integrity (Tottoli *et al.*, 2020).

Wounds healing is an acute inflammatory response which involved an interaction of cytokines, growth factors, chemokines and chemical mediator from cells to restore and replace the integrity of the skin. This process is said to be complex biological process where it consists of complex interaction of extracellular matrix (ECM), soluble mediators, various resident cells; fibroblasts and keratinocytes, and infiltrated leukocyte subtypes (Pereira Beserra *et al.*, 2019). Wound healing process is classified to four overlapping and codependent phases which start with the hemostasis or coagulation phase, the inflammatory phase, the proliferative phase, and the remodeling phase (Figure 2.2) (Pauzi *et al.*, 2018). The wound healing mechanism reinstate tissues integrity however, it is limited to only wound repair in certain cases (Tottoli *et al.*, 2020). The tissue that actually heals and restore their integrity and function is solely bone. Other wounds would heal to some extent as it left degrees of collagenous scar formation which undergoes maturation in the process and at the same time will never return to its original structure and somehow hinder the original function of the tissue (Melrose, 2016). Besides, if the wounds are not treated as they should be, they can become chronic or progressively fibrotic which cause impairment in tissue function in an instance lead to organ failure and in worst case can lead to death (Pereira Beserra *et al.*, 2019).

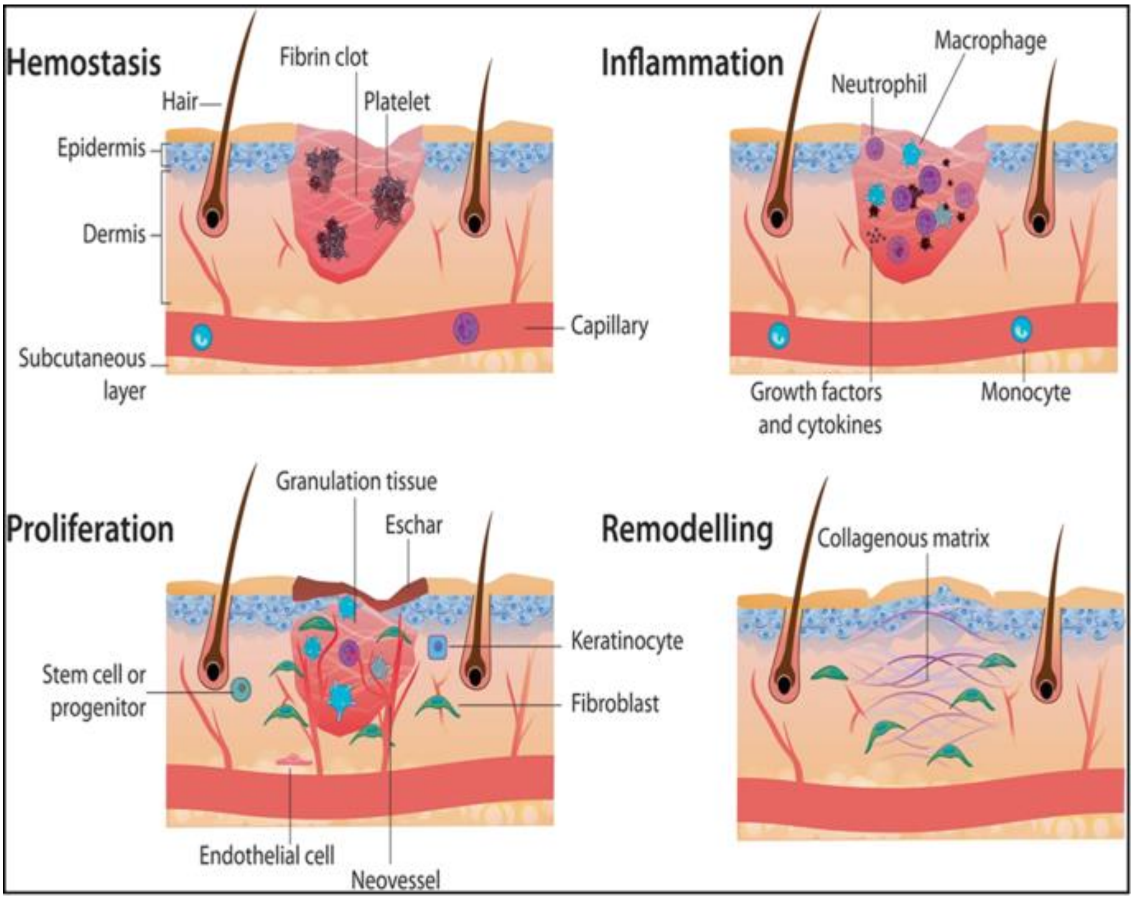


Figure 2.2 Wound healing phases adapted from (Nour *et al.*, 2019)

2.3.1 Hemostasis Phase

This wound healing phase is initiated when tissue injuries occurred. This occurrence leads to this first phase of wound healing clinically referred as the homeostasis or bleeding phase. The wound exposing the collagen to surrounding and immediately initiate the activation of intrinsic and extrinsic clotting cascade (Sinno & Prakash, 2013). Thromboxane A2 and prostaglandin 2-alpha also activated, resulted in vascular constriction and platelet aggregation responses at the site of injury (Ghatak *et al.*, 2015; Sinno & Prakash, 2013). Damage endothelium secretes vasoconstrictors known as endothelin which contributes to reflexive contracture of vascular smooth muscle (Rodrigues *et al.*, 2019). In addition, the bleeding that occurs help in removing antigens and pathogens while keratinocytes secrete Interleukin-1 (IL1) and platelets secrete hemostatic factors produce fibrin network or fibrin clots (Nour *et al.*, 2019). The first 1 hour of injury, the production of platelet factors is enormous and continue to be released by activated platelets up to 7 days. In this stage, macrophages act as microbicidal and pro-inflammatory that produce TNF-α, IL-6 and IL-1β and often referred as M1(Rodrigues *et al.*, 2019). M1 macrophages responsible for phagocytic activity, scavenging and production of pro-inflammatory cells (Sorg *et al.*, 2017). The presence of various bacteria and pro-inflammatory factors such as tumor necrosis factor-α (TNF-α) enhance and stimulate the production of chemokines release. Furthermore, mast cells can also be found immediately after injury to assemble inflammatory cell and also involved in allergic response (Rodrigues *et al.*, 2019).

In this phase, the formation of fibrin clots are at the center of attention as they consist of many important molecules secreted by platelets and monocytes such as fibronectin (FN), Secreted Protein, Acidic and Rich in Cysteine (SPARC), thrombospondin, vitronectin and growth factors; transforming growth factor (TGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), epidermal growth factor (EGF) and insulin-like growth factor-1 (IGF-1) (Ghatak *et al.*, 2015). The fibrins clot is created to reduce bleeding from damage of microvasculature (Rodrigues *et al.*, 2019) and provide preliminary matrix for inflammatory cells to infiltrate and migrate into the side of injury for further wound repair process (Ghatak *et al.*, 2015; Nour *et al.*, 2019). In an instance, the production of inflammatory interleukins and cytokines, in the present of exudates; rich protein, histamine and serotonin help in making way for inflammatory cell such as monocytes to easily penetrate to the wound area to start the phagocytosis activity by increasing the permeability of blood vessels (Nour *et al.*, 2019), besides forming a barrier to fight against the breaching of external microorganisms (Gonzalez *et al.*, 2016).

2.3.2 Inflammatory Phase

Fibrin clot controlled the bleeding at the injury area, right then abundant of responsible cells continuously infiltrate into the site of injury and stimulate the second phase of wound healing known as inflammatory phase. The responsible inflammatory cells such as neutrophils, macrophages, and lymphocytes infiltrate into the wound for further healing process. The infiltration of various inflammatory cells into the wound sites is pathognomic known as chemotaxis. Neutrophils are the first inflammatory cells that infiltrate to the site of wound as they kill every microbes, bacteria and cellular debris that storm into that area (Ghatak *et al.*, 2015) by setting off toxic granules, producing an oxidative burst, starting phagocytosis and creating neutrophil extracellular traps known as NETs (Rodrigues *et al.*, 2019). They kill the two birds with one stone by acting as microbe's killer and at the same time promote wound healing process (Pereira Beserra *et al.*, 2019). Besides, neutrophils can produce protease and reactive oxygen species (ROS) (Ghatak *et al.*, 2015); if produced in large number they can contribute to more damage to the tissue and lead to non-healing wound. In other words, the prolong stimulation and excessive production of neutrophil can lead to worst case of wound (Chronic wound) thus, the recruitment of macrophages are compulsory to eliminate extended and excess of neutrophil to avoid further destruction of wound tissue (Ghatak *et al.*, 2015; Pereira Beserra *et al.*, 2019).